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Article

Spontaneous Lytic Replication and Epitheliotropism Define an Epstein-Barr Virus Strain Found in Carcinomas

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SUMMARY

The Epstein-Barr virus (EBV) is found in a variety of tumors whose incidence greatly varies around the world. A poorly explored hypothesis is that particular EBV strains account for this phenomenon. We report that M81, a virus isolated from a Chinese patient with nasopharyngeal carcinoma (NPC), shows remarkable similarity to other NPC viruses but is divergent from all other known strains. M81 exhibited a reversed tropism relative to common strains with a reduced ability to infect B cells and a high propensity to infect epithelial cells, which is in agreement with its isolation from carcinomas. M81 spontaneously replicated in B cells in vitro and in vivo at unusually high levels, in line with the enhanced viral replication observed in NPC patients. Spontaneous replication and epitheliotropism could be partly ascribed to polymorphisms within viral proteins. We suggest considering M81 and its closely related isolates as an EBV subtype with enhanced pathogenic potential.

INTRODUCTION

The Epstein-Barr virus (EBV) chronically infects the large majority of the world population, usually without clinical consequences. However, in a minority of individuals, EBV infection is associated with the development of a variety of diseases, ranging from infectious mononucleosis (IM) syndromes to various cancers (Rickinson and Kieff, 2007). Although the virus homogeneously targets populations from all geographic areas, the incidence of the diseases it causes can vary drastically (Chang et al., 2009). While IM is common in Western populations, Burkitt lymphoma (BL) is endemic in equatorial Africa and nasopharyngeal carcinoma (NPC) is one of the most common tumors encountered in Southeast Asia. What causes these variations in disease incidence is unclear, although the age at which EBV infection takes place, environmental factors such as food contaminations with nitrosamines and phorbol esters or smoking, and concurrent diseases such as malaria are all known to play an important role (Hsu and Glaser, 2000; Hsu et al., 2009; Jia and Qin, 2012; Yu and Yuan, 2002). The genetic background of the affected individuals has also been invoked to explain this puzzling phenomenon (Hildesheim et al., 1997; Levine et al., 1992; Li et al., 2009; Lu et al., 1990; Ung et al., 1999). Another nonexclusive hypothesis is that the occurrence of these diseases reflects the existence of multiple virus subtypes that are endowed with different properties but are found only in restricted geographic areas. Strong evidence for genetic polymorphisms between EBV isolates has been garnered from partial or total sequencing of multiple viruses from all over the world. However, it has not been easy to appreciate the consequences of this genetic heterogeneity in terms of viral functions, particularly because of the difficulty in assessing the biological properties of viruses that carry these various polymorphisms. Here, we report an attempt at linking the genetic polymorphisms found in these viruses with their phenotypic traits. We have cloned and fully sequenced M81, a virus isolated from an NPC that developed in a Chinese individual (Desgranges et al., 1976, 1979). We found that M81 is highly similar to viruses isolated from NPCs and that it profoundly differs in terms of virus infection and replication from extensively characterized Western strains. These observations demonstrate the existence of distinct EBV subtypes and suggest that the unusual properties evinced by M81-type viruses are likely to explain their tight association with NPC.

RESULTS

The M81 Strain Is Representative of Viruses that Infect NPC Tissues

In an attempt to study the genotype and phenotype of the EBV strain M81, we cloned its genome onto the F-factor-based

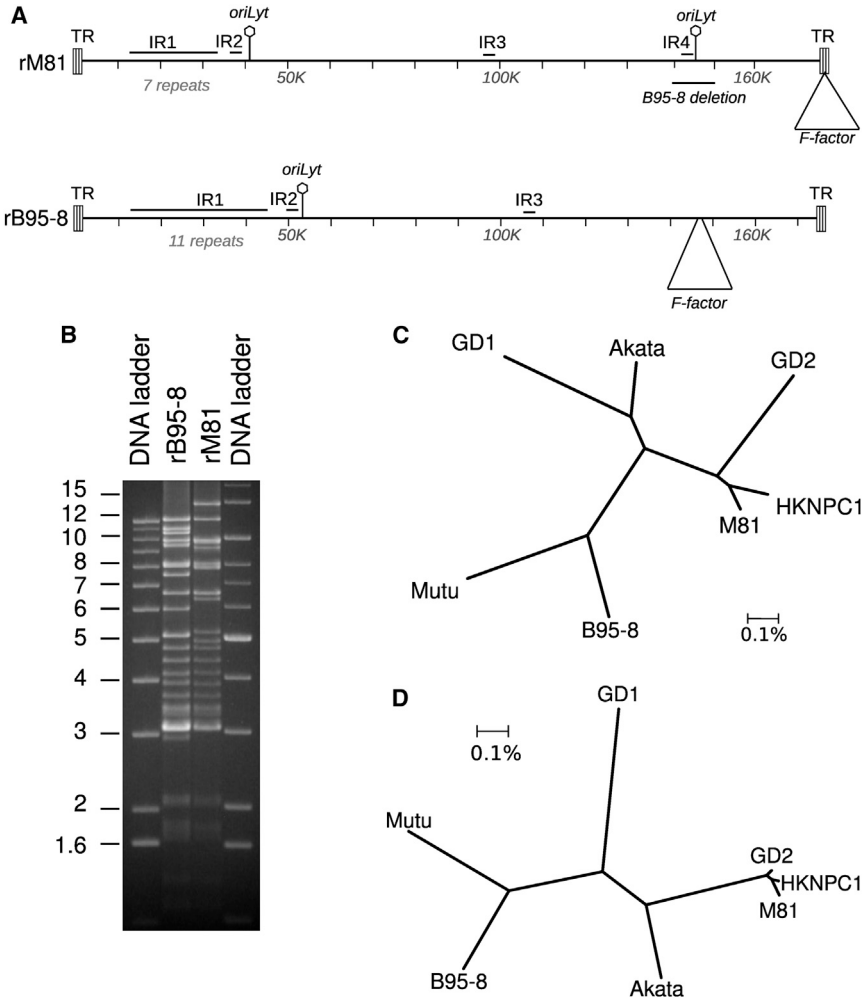

Figure 1. Generation and Characterization of the Recombinant M81 EBV Genome

(A) Genomic maps of the recombinant M81 (rM81) and B95-8 (rB95-8) viruses. The rM81 was generated from the marmoset M81 cell line by inserting an F-factor plasmid coupled to the hygromycin resistance cassette between the terminal repeats (TR). IR, internal repeats.

(B) Minipreparations of DNA from the rM81 and the rB95-8 genome were cleaved with the restriction enzyme BamHI.

(C and D) Distance trees based on available EBV genome sequences at the DNA (C) and protein level (D) show that the M81 strain is closely related to GD2 and HKNPC1, two EBV strains found in NPC tumor cells.

See also Figures S1 and S2.

replicon in *E. coli* as described before (Delecluse et al., 1998). The F-factor-based replicon was recombined into the terminal repeats and therefore did not disrupt coding regions (Figure 1A). The structure of the recombinant M81 strain genome (rM81) was assessed by restriction enzyme analysis (Figure 1B). This analysis revealed that the M81 genome is larger than B95-8 but also that multiple genomic polymorphisms exist between the two strains, as the length of the fragments was frequently different. In parallel, we completely sequenced the rM81 genome to allow its classification among sequenced EBV strains as well as its polymorphisms. To this end, we built genetic distance trees based on the published type 2A EBV genomes that include B95-8, one of the prototypical laboratory strains (Baer et al., 1984), the Akata and the Mutu viruses that were isolated from BLs that respectively developed in a Japanese and an African patient (Gregory et al., 1990; Lin et al., 2013; Takada et al., 1991), as well as GD1, GD2, and HKNPC1, three viruses that were either rescued from Chinese NPC patients with NPC or directly isolated from the tumors (Kwok et al., 2012; Liu et al., 2011; Zeng et al., 2005). The nonsequenced gene regions of the HKNPC1 genome (6,479 nt; coordinates 35,273–36,144, 38,194–40,624, and 139,951–143,126) and the region corresponding to the BART deletion in B95-8 could not be included into the analysis. We further trimmed the terminal and BamHI-W repeats to have the same number of repetitions across all sequences. We found that M81 is closely related to the other NPC strains but much more distant from the other strains (Figures 1C and 1D). In particular, M81 and HKNPC1 showed only 0.2% divergence at the DNA level (Figure 1C). This is important because HKNPC1 was directly sequenced from an NPC biopsy specimen and was therefore present in the tumor cells (Kwok et al., 2012). We refined our analysis by comparing 50 viral protein sequences among these strains and found that M81 is 99.9% homologous to HKNPC1 but much more distant from B95-8 (Figure 1D). Additional distance trees are shown in Figure S1 for key EBV proteins such as LMP1, EBNA2, BZLF1, and BLLF1. We conclude that M81 is a valid representative of the EBV strains that are found in Chinese NPC. We then attempted to extend our comparisons to EBV isolates found in the general population. Numerous studies have compared the sequence of the LMP1 gene and of the *BZLF1* gene and its promoter among isolates worldwide (see Table S1). This has led to the recognition of an EBV variant preferentially, but not exclusively, found in Asian countries. We found that M81 belongs to this variant whose frequency increases from North to South China and that is preferentially found in NPC (Table S1). These results suggest that rM81 can be used to study the properties of this variant isolate. However, the limited number of genes on which the recognition of the variant is based may not account for the complexity provided by the whole virus, and it is more likely that the variant category of EBV comprises multiple virus isolates with variation in properties.

We then stably introduced the rM81 DNA into human embryonic kidney 293 (HEK293) cells to generate a virus producer cell line. The integrity of the EBV genome in the stably transfected

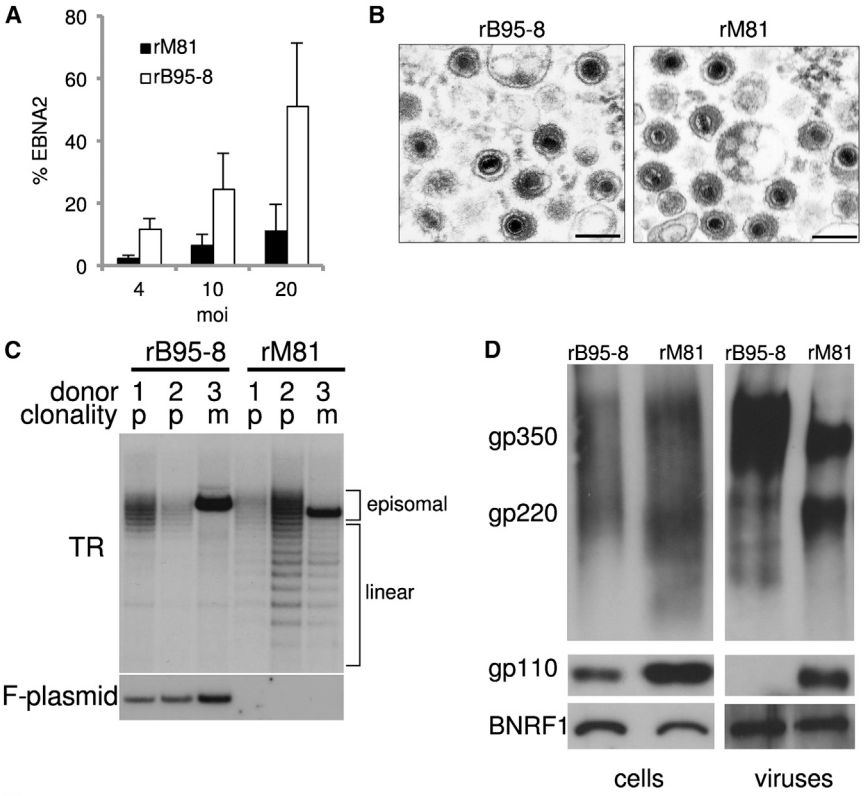

Figure 2. M81 Displays a Reduced Tropism for B Cells but M81 and B95-8 Do Not Differ in Their Transforming Potential

(A) Primary human B cells were exposed to the same number of viral genomes and infected cells were stained for EBNA2 3 dpi. Values are represented as mean ± SD (B) rM81 and rB95-8 present in infectious supernatants from induced producer cells were pelleted by ultracentrifugation and analyzed by transmission electron microscopy. Bar: 200 nm.

(C) Southern blot analysis of BamHI-cleaved genomic DNA isolated from LCLs generated with B cells from three different donors after infection with rB95-8 or rM81. The DNAs were hybridized with a probe specific for the terminal repeats (upper panel) or for the F-plasmid (lower panel). p, polyclonal culture; m, monoclonal culture.

(D) Western blot analysis performed with antibodies specific to the viral glycoproteins gp350/220 and gp110 on proteins extracted from lytically induced producer cell lines or their pelleted supernatants. An antibody specific to the tegument protein BNRF1 was used as a loading control.

(E) Alignment of the BLLF1 gene that encodes gp350 from either M81 or B95-8. Three major deletions in M81 BLLF1 are shown.

(F) A transformation assay with rM81 or rB95-8 was performed with a similar number of EBNA2 infected cells per well. The transformation efficiency was analyzed by determining the percentage of outgrowing colonies 30 dpi.

Values represent mean ± SD. See also Figures S3 and S4.

HEK293 clones was confirmed by shuttling the rM81 episomes they contained into *E. coli* cells from which restriction analyses could be performed (Figure S2). We then activated virus production by transfecting *BZLF1* into the producer cells and used the generated viruses to determine rM81's phenotypic traits.

M81 Has a Reduced B Cell Tropism Relative to B95-8, but M81 Efficiently Transforms Infected B Cells

We began assessing M81's tropism by infecting resting primary peripheral blood B cells. Three days after exposure to supernatants containing the same genome copy numbers of rM81 or rB95-8, the B cells were stained with an antibody specific to the viral nuclear antigen EBNA2. This assay revealed that the number of infected cells after incubation with rM81 was between five and ten times lower than after exposure to rB95-8 (Figure 2A), although viruses in pelleted supernatants from both producer cell lines were visible in both cases and evinced similar morphological traits (Figure 2B). Similar results were obtained with M81 viruses collected from the original marmoset M81 LCL, demonstrating that rM81's reduced B cell tropism was not due to a cloning artifact (Figure S3). Importantly, we found that B cells infected with rM81 exclusively carried viral episomes that were devoid of the F-plasmid but conserved a large number of terminal repeats (Figure 2C). This can be explained by excision of the F-plasmid through recombination of the highly homologous terminal repeats that flank it, as previously described for another recombinant herpesvirus genome (Zhou et al., 2010). We then wished to determine whether these results could be explained by a reduced ability of M81 to bind to B cells. We exposed B cells to supernatants containing the same genome equivalents of rM81 or rB95-8, extensively washed the cells, and determined by quantitative PCR (qPCR) the number of viruses bound to the surface of the cells. We found that rM81 and rB95-8 had a very similar binding efficiency to B cells (data not shown).

EBV infects B cells through interaction of viral glycoproteins with cellular receptors (Connolly et al., 2011; Hutt-Fletcher, 2007). Among these glycoproteins, gp350 and gp110 play an important role for binding to and for fusion with the B cell membranes. Therefore, we assessed the amount of gp350 in M81 and

B95-8 virions by western blotting using specific antibodies. The results of these assays, shown in Figure 2D, revealed that gp350 is expressed in producer cell lines and free virions at approximately the same level. However, western blotting showed that M81 gp350 migrates quicker than its homolog in B95-8 and that M81 gp220, the spliced form of gp350, is more abundant relative to its B95-8 homolog. Analysis of M81's sequence revealed multiple deletions in the *BLLF1* gene that encodes this protein relative to B95-8 that could account for the difference in protein size (Figure 2E). Therefore, we complemented a rB95-8 devoid of the *BLLF1* gene with an expression plasmid carrying the *BLLF1* gene encoded by M81 or B95-8 and assessed its ability to bind to and to infect B cells. Complementation significantly enhanced binding and infection of B cells but we could not identify any difference after complementation with either type of *BLLF1* (data not shown). Thus, M81 demonstrates a reduced B cell tropism, which, however, is not caused by its altered gp350 sequence.

We then determined the ability of rM81 to transform B cells. Resting B cells were exposed to rB95-8 or rM81 at multiplicities of infection (MOIs) that gave rise to the same number of EBNA2-positive cells. Infected B cells were seeded at low cell density in 96-well plates, and we counted the number of outgrowing colonies 30 days postinfection (dpi). We found that the number of colonies, and therefore the ability to initiate B cell transformation, was similar with both viruses (Figure 2F). Similar experiments performed with nonrecombinant viruses had the same outcome (Figure S3).

B Cells and 293 Cells Infected by M81 Evince Enhanced Lytic Replication In Vitro

We quantified virus production in the 293/rM81 producer cell lines using qPCR with primers and probes specific for the nonrepetitive sequence that encodes the viral DNA polymerase. We found that all studied clones produced virus at high levels, on average five times higher than 293/rB95-8 cells that carry the B95-8 recombinant genome, although the number of replicating cells as determined by gp350 staining was identical (8.3 versus 7.7%, respectively) (Figure 3A; data not shown). This more efficient virus production in 293/rM81 does not correlate with a higher EBV copy number within the stably transfected 293 cells (Figure 3B). We then studied virus production in LCLs established from 18 healthy donors of European or Asian descent with the rM81, rB95-8, or rAkata EBV strains. Surprisingly, all LCLs generated with rM81, but none of those infected with rB95-8 or with rAkata, showed evidence of spontaneous lytic replication. This included robust expression of the immediate early protein BZLF1 beginning at around 18–20 dpi, expression of the late structural protein gp350 on most cells (Figure 3C), and production of free viruses in the supernatant as defined by qPCR (Figure 3D). However, the gp350 signals were confined to the cell surface and only a minority of cells displayed intense cytoplasmic staining as expected from virus-producing cells. This staining pattern is most likely due to virus binding to the surface of nonreplicating B cells. We then monitored virus replication in LCLs infected with rM81 for more than 3 months using BZLF1 expression as a readout and found that rM81 LCLs established with different donors undergo a persistent and robust lytic replication for the whole study period (Figure 3E). These cells also kept producing gp350 at high levels (data not shown). Viral DNA replication and production of progeny virus (Figure 2C) could be validated by a Southern blot assay detecting newly generated linear viral genomes and by the detection of newly produced viruses in electron micrographs, both in lymphoid cells and in the supernatant (Figure 3F). We could also propagate the infection from B cells to B cells by infecting resting B cells with supernatants from LCLs generated with rM81 (data not shown). Importantly, these results could be repeated with the nonrecombinant M81, demonstrating again that the cloning procedure was not responsible for rM81's properties (data not shown).

Polymorphisms in the BZLF1 Protein Contribute to Spontaneous Lytic Replication in LCLs Transformed with rM81

The different outcome after infection of the same B cells with rB95-8, rAkata, or rM81 prompted us to search for polymorphisms between M81 and B95-8. One obvious difference between M81 and B95-8 is the absence of some of the BART microRNAs (miRNAs) in the latter. However, most EBV strains carry these genetic elements and none of them were previously reported to spontaneously replicate as strongly as M81 (Kieff and Rickinson, 2007). Indeed, B cells infected with Akata, which carries all BART miRNAs, showed only weak and transitory lytic replication (Figure 3C; data not shown). Another obvious candidate was BZLF1, the lytic replication master protein (Miller, 1990). Therefore, we constructed a recombinant M81 genome that lacks the genome fragment that carries the *BZLF1* gene. We infected B cells with this M81/ΔBZLF1 virus and with a B95-8/ΔBZLF1 virus that we previously reported (Feederle et al., 2000). The resulting LCLs were then stably transfected with an expression plasmid encoding the M81/*BZLF1* or the B95-8/*BZLF1* genes, under the control of a doxycycline-inducible promoter. We found that lytic replication can be efficiently induced in LCLs transformed by M81/ΔBZLF1 and complemented by the M81/*BZLF1* gene but that this complementation was much less efficient with B95-8/*BZLF1* (Figure 4A). However, M81/BZLF1 was not better than its B95-8 homolog in its ability to induce lytic replication in B95-8/ΔBZLF1 LCLs. We conclude that polymorphisms in the BZLF1 genes partly explain M81's ability to induce lytic replication but that other viral genes play an important role in the ability of the B cells to support lytic replication.

We then addressed the possibility that the spontaneous lytic replication we observed in cells infected with M81 was the result of a polymorphism in the promoter of *BZLF1*. Indeed, this region was previously found to be highly polymorphic, with some polymorphisms being typically observed in Chinese strains (Jin et al., 2010; Luo et al., 2011). Therefore, we constructed an M81 recombinant virus in which the *BZLF1* gene and its promoter were exchanged against its homolog from B95-8. Monitoring of BZLF1 expression in cells infected by this mutant showed spontaneous expression 21 dpi, as did the wild-type rM81 virus (Figure 4B). We conclude that the initiation of spontaneous replication is not due to the differences in the exchanged regions between B95-8 and M81.

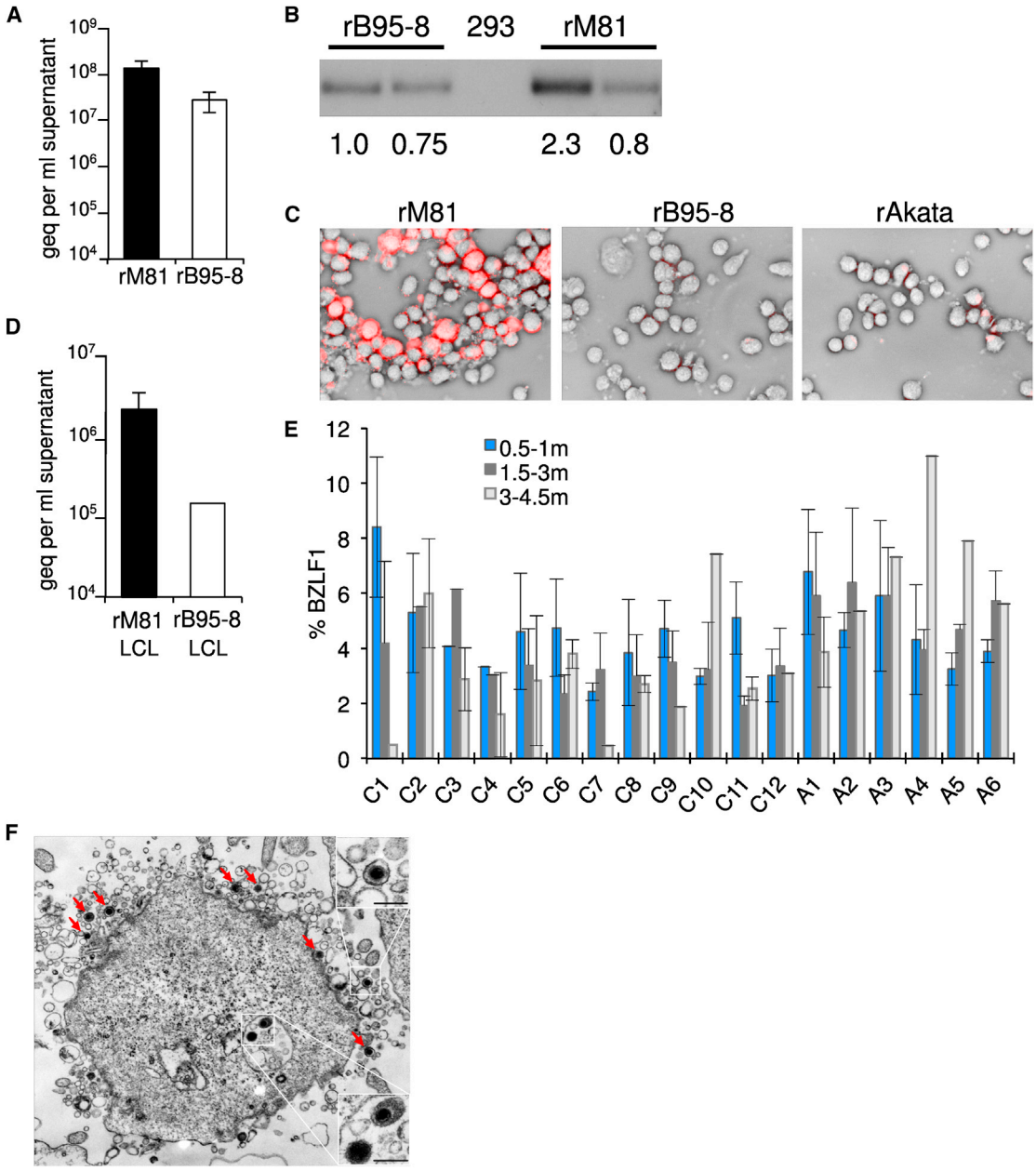

Figure 3. Primary Human B Cells Infected with M81 Show Enhanced Lytic Replication

(A) The figure displays the viral titers measured in supernatants of induced rM81 or rB95-8 producer cell lines. Values represent mean ± SD. geq, genome equivalents.

(B) A Southern blot analysis of genomic DNAs extracted from different producer cell lines was performed with a nonrepetitive EBV-specific probe spanning the *BMRF1* fragment. The intensity of the signals is given with the first signal normalized to one.

(C) LCLs were generated by infecting B cells from the same donor with rM81, rB95-8, or rAkata. These continuously growing cell lines were stained at 44 dpi with an antibody specific to gp350. Overlayed and inverted pictures are presented.

(D) Viral titers in supernatants from noninduced LCLs transformed with rM81 or rB95-8 were determined by qPCR. The results of this assay are given as genome equivalents per ml of supernatant. Values represent mean ± SD.

(E) LCLs generated with B cells from 18 independent donors (12 whites, C1-C12; 6 Asian donors, A1-6) were infected with rM81 and monitored for virus production for up to 4.5 months. Immunostaining for BZLF1 was used as a readout for spontaneous lytic replication and the percentage of cells positive for this protein are given. The graph of bars gives the mean ± SD of weakly measurements performed during the indicated period of time in months (m).

(F) Virus production in rM81-transformed LCLs and pelleted supernatants thereof was examined by transmission electron microscopy. Arrows highlight virus particles. Bar in insets represents 200 nm.

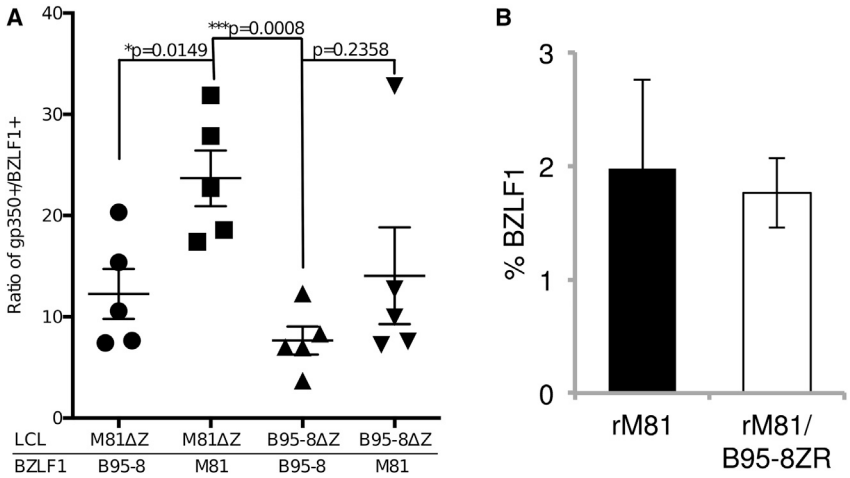

Figure 4. Polymorphisms within the BZLF1 Protein Encoded by M81 Contribute to the Strong Lytic Replication Observed in LCLs Transformed by rM81

(A) LCLs were generated by exposure to rM81/ΔBZLF1(M81ΔZ) or rB95-8/ΔBZLF1(B95-8ΔZ) and were stably transfected with an expression plasmid encoding the *BZLF1* gene encoded by either M81 or B95-8. These genes are under the control of a tetracycline-inducible promoter. The ratio of gp350-positive relative to BZLF1-positive cells 7 days postdoxycycline treatment is indicated. For each of the indicated conditions, five experiments were performed within 2 months postinfection with LCLs from two donors. The statistical significance of the findings is given.

(B) LCLs were generated by infection with wild-type rM81 or with a recombinant rM81 virus that carries the genes *BZLF1* and *BRLF1* from B95-8 (B770). The expression rate of the lytic proteins BZLF1 and gp350 in these LCLs 30 dpi is indicated. Values represent mean ± SD of two measurements from two LCLs generated with two different donors.

rM81's Phenotypic Traits in B Cells Can Be Confirmed in an Animal Model

We wished to determine whether M81's properties observed in vitro after B cell infection could be reproduced in vivo. To this end, we infected NSG-A2 mice, whose immune system had been reconstituted with human hematopoietic progenitor cells (Rämer et al., 2011; White et al., 2012). Altogether, 13 mice were inoculated intraperitoneally with either rB95-8 (7 mice) or rM81 viruses (6 mice) in two independent experiments, monitored for peripheral blood viral load, and then euthanized to record histological evidence of infection and T cell expansion. Five control mice were mock-treated with PBS. We found that in all infected mice, EBV infection could be documented longitudinally by measuring viral loads in the blood and assaying expression of the EBV-specific noncoding RNAs EBER in spleen sections at the termination of the experiments (Figure 5). We also observed $CD8^+$ T cell expansion and upregulation of the activation marker HLA-DR on this T cell subset during the course of infection (Figure 5A; data not shown). In one experiment, we exposed the mice to supernatants containing the same number of rB95-8 or rM81 genomes. In that case, we noticed that the number of B95-8-infected B cells in the spleen was much higher than the number of those infected by rM81 in all investigated mice (Figure 5A). In some mice, additional organs such as the kidney contained EBV-positive cells in rM81-infected mice. Quantification of EBV copies in the peripheral blood of infected animals confirmed the increased viral load after infection with rB95-8 (Figure 5B). These results are in line with M81's reduced B cell tropism observed in vitro (see Figure 2A). In another experiment, we inoculated the mice with virus doses of either rM81 or rB95-8 that gave rise to the same number of infected B cells in vitro, as assessed by an EBNA2-specific staining 3 dpi. In that case, similar numbers of EBV-positive B cells were visible in the spleen of animals infected with either type of virus (Figure 5C). We also found rM81- and B95-8-infected B cells in the mesenteric lymph nodes in comparable numbers (Figure 5C). However, the viral load in the blood became much higher after infection with rM81 4 weeks postinfection (Figure 5D). These results suggested either that a larger number of infected B cells circulated in the blood after infection with rM81, or that a high degree of lytic replication was taking place in the tissues of infected animals. We therefore stained tissues from infected mice with antibodies specific to the early and late replication proteins BZLF1 and gp350. In all cases, mice infected with rM81 carried many more B cells undergoing lytic replication than rB95-8 infected animals (Figure 5E). Indeed, numerous cells expressed the BZLF1 or the gp350 lytic proteins after rM81 infection in vivo (Figure 5E). This was particularly pronounced in the second experiment after inoculation of rM81 at higher doses (Figure 5E). All mice infected with rM81, either at low or high doses, showed evidence of lytic replication in tissues that carried EBV-infected cells. In contrast, we could not find any unambiguously gp350-positive B cells in any of the mice infected with rB95-8, although a few BZLF1-positive B cells were visible in the spleen of one mouse (Figure 5E). We conclude that rM81 leads to virus replication in infected B cells in vivo even in the presence of human immune system activation.

Enhanced Tropism for Epithelial Cells

The isolation of M81 from an NPC implies that this viral strain has the ability to infect epithelial cells. We and others have previously found that EBV strains generally have a very weak tropism for primary epithelial cells with infection rates of less than a few cells per thousand, except if viruses are forced to express high amounts of gp110 and highly differentiated epithelial cells are infected (Feederle et al., 2007; Shannon-Lowe and Rowe, 2011; Shannon-Lowe et al., 2006). We exposed primary epithelial cultures (pEC) from the epithelium that covers the sphenoidal sinus, because this mucosa shares many histological features with the nasopharynx epithelium. We previously showed that resting B cells can be used as virus carriers for epithelial cell transfer infection with viruses that incorporated large amounts of gp110 into the virus envelope (Shannon-Lowe et al., 2006). Therefore, we performed cell-free as well as transfer infections with pECs using

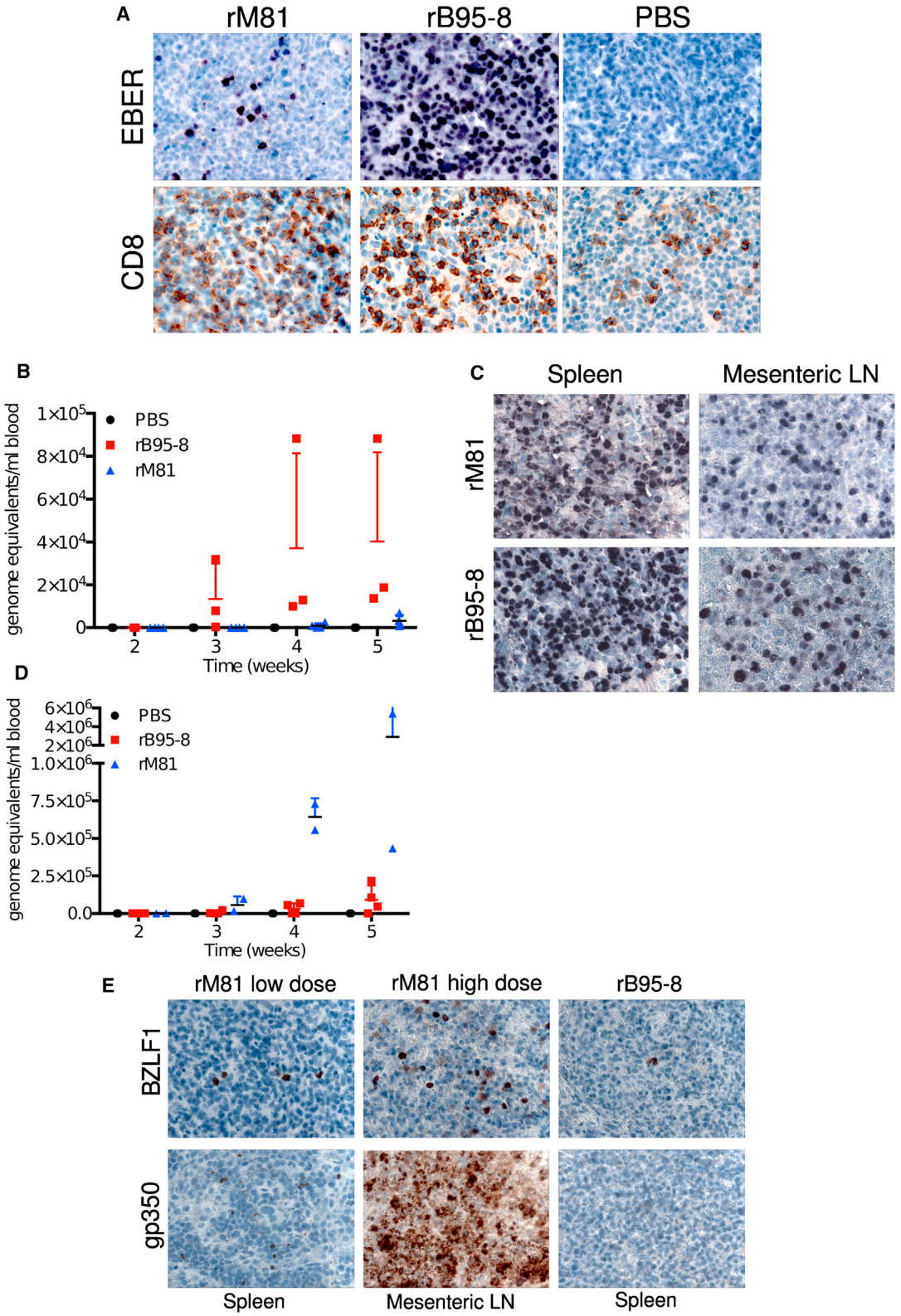

Figure 5. Elevated Lytic EBV Replication in M81-Infected Mice with Reconstituted Human Immune System Components

(A) The pictures show an in situ hybridization with an EBER-specific probe performed on histological sections from the spleens of animals exposed to the same number of genomes from either rB95-8 or rM81 or mock-infected with PBS. These sections were also stained with an antibody specific to CD8.

(legend continued on next page)

100 rM81 or rB95-8 genome equivalents per target cell and stained treated cells for keratin and for EBER expression (Figures 6A and 6B). We found that all infected cells after transfer infection were keratin positive and thus infected epithelial cells. The results of both types of infections are presented in Figure 6C and show that cell-free rB95-8 can hardly or not at all infect pECs at an intermediate (passage 6) to late (passage 9) passaging number, which corresponds to a intermediate differentiation state (Feederle et al., 2007). In contrast, we observed rates of infection that ranged between 3% and 7% after infection with cell-free rM81. Higher infection rates, ranging from 4% to 26%, were obtained after transfer infection with rM81, but transfer infection with rB95-8 remained inefficient in all performed experiments (Figure 6C). We have previously shown that the glycoprotein gp110 is important for infecting primary epithelial cells and that B95-8 contains low amounts of this protein (Neuhierl et al., 2002). Therefore, we performed a western blot analysis for gp110 and found that this protein was expressed at higher levels in the M81 producer cells and in the released virions than in their B95-8 counterparts (Figure 2D; Figure S4).

Therefore, we repeated these experiments with viruses obtained from producer cell lines complemented with a *BALF4* expression plasmid to increase gp110's concentration in the produced viruses and found that the rate of cell-free infection increased for both rB95-8 and rM81, although the enhancing effect was much more pronounced for the latter viruses (Figure 6D). Transfer infection with these gp110-complemented viruses had little influence on the outcome of infection; infections with rB95-8 remained essentially negative while those with rM81 were concordant with experiments performed with noncomplemented viruses. We then tested whether complementation with gp110 from M81 was more efficient than those with gp110 from B95-8 by complementing virus-producing cell lines with either source of protein. We found that complementation with gp110 from M81 was more efficient relative to complementation with gp110 from B95-8 (Figure 6E). We conclude that polymorphisms within gp110 and its expression level partly explain M81's strong epitheliotropism but other factors remain to be discovered.

We then studied the outcome of infection in epithelial cell and found that many infected cells expressed BZLF1 (Figure 6F). A subset of these also expressed gp350 and produced viruses visible in electron microscopy, demonstrating that infected cells complete lytic replication (Figure 6G). Thus, M81 infects epithelial cells much more efficiently than B95-8 and also establishes spontaneous lytic replication in a subset of infected epithelial cells.

DISCUSSION

This study reports an extensive comparative characterization of a virus isolated in a NPC developed by a Chinese patient and how this virus differs from viruses isolated in other parts of the world. Although M81 and B95-8 can both infect epithelial cells, the first strain is predominantly epitheliotropic and the second B lymphotropic. We identified the abundance of gp110 as an important factor in the ability of M81 to infect epithelial cells. Nevertheless, other viral proteins are evidently implicated in this viral function. We previously reported that B95-8 complemented with gp110 can infect differentiated primary epithelial cells with high efficiency (Feederle et al., 2007). M81 fundamentally differs from B95-8 in that it infects epithelial cells at intermediate passage number and differentiation stage in the absence of any complementation and with a much higher efficiency. Telomerase-immortalized cell lines established from epithelial cells of the nasopharynx that carry genetic abnormalities such as the loss of p16 have been found to be more sensitive to EBV infection (Tsang et al., 2010). These observations could explain the difficulty encountered by many groups to infect normal epithelial cells. However, a more recent paper from the same group made clear that p16 downregulation or Cyclin D upregulation facilitates virus persistence rather than virus infection (Tsang et al., 2012). These data concur with our observations that the viral subtype rather than the chromosomal integrity determines the efficiency of infection. Spontaneous lytic replication in LCLs is in the large majority of cases transitory and limited in intensity (Kieff and Rickinson, 2007). In contrast, we see extensive late protein and virus production in all types of cells infected with rM81. Interestingly, the only report of spontaneous lytic replication in LCLs generated with adult primary B cells was after infection with ABA, a virus isolated from an NPC that developed in an African patient (Crawford et al., 1979). We found that the increased and sustained lytic replication in B cells infected by M81 is partly due to polymorphisms within the BZLF1 protein but that other so-far-unknown genetic elements have a strong influence on this crucial viral function. In contrast, polymorphisms within BZLF1's promoter that have been previously described do not seem to play an important role in M81's ability to spontaneously initiate replication. Here again, other unknown genetic elements that govern M81's ability to replicate remain to be identified. Importantly, mice carrying reconstituted human immune system components also displayed high levels of lytic replication after infection with rM81, irrespective of the overall viral loads and the numbers of EBV-infected B cells they carry. Further studies need to determine whether the observed T cell expansion after rM81 infection that is presumably driven by lytic EBV antigens provides protection against viral replication, as has been previously shown for rB95-8 infection with a higher frequency of latent over lytic EBV infection (Strowig et al., 2009).

M81 was isolated in a geographic area where NPC occurs at high incidence and this virus is genetically very close to other viruses present in NPC cells. Patients with NPC carry viruses

(B) Dynamics of virus load in the peripheral blood of mice infected under conditions described in (A); rB95-8 viral load in the peripheral blood was significantly higher than M81 virus load at week 5 (P value is 0.0009).

(C) Reconstituted mice were exposed to viral titers that lead to the infection of the same number of B cells, as defined by EBNA2 staining, or mock infected. In situ hybridization with an EBER-specific probe was performed on splenic tissues and on mesenteric lymph nodes tissues.

(D) Dynamics of virus load in the peripheral blood of infected mice infected under conditions described in (C); rM81 viral load was significantly higher than rB95-8 virus load at week 5 (P value is < 0.0001).

(E) Immunostains for BZLF1 and gp350 were performed on tissues of mice infected with rM81 or rB95-8.

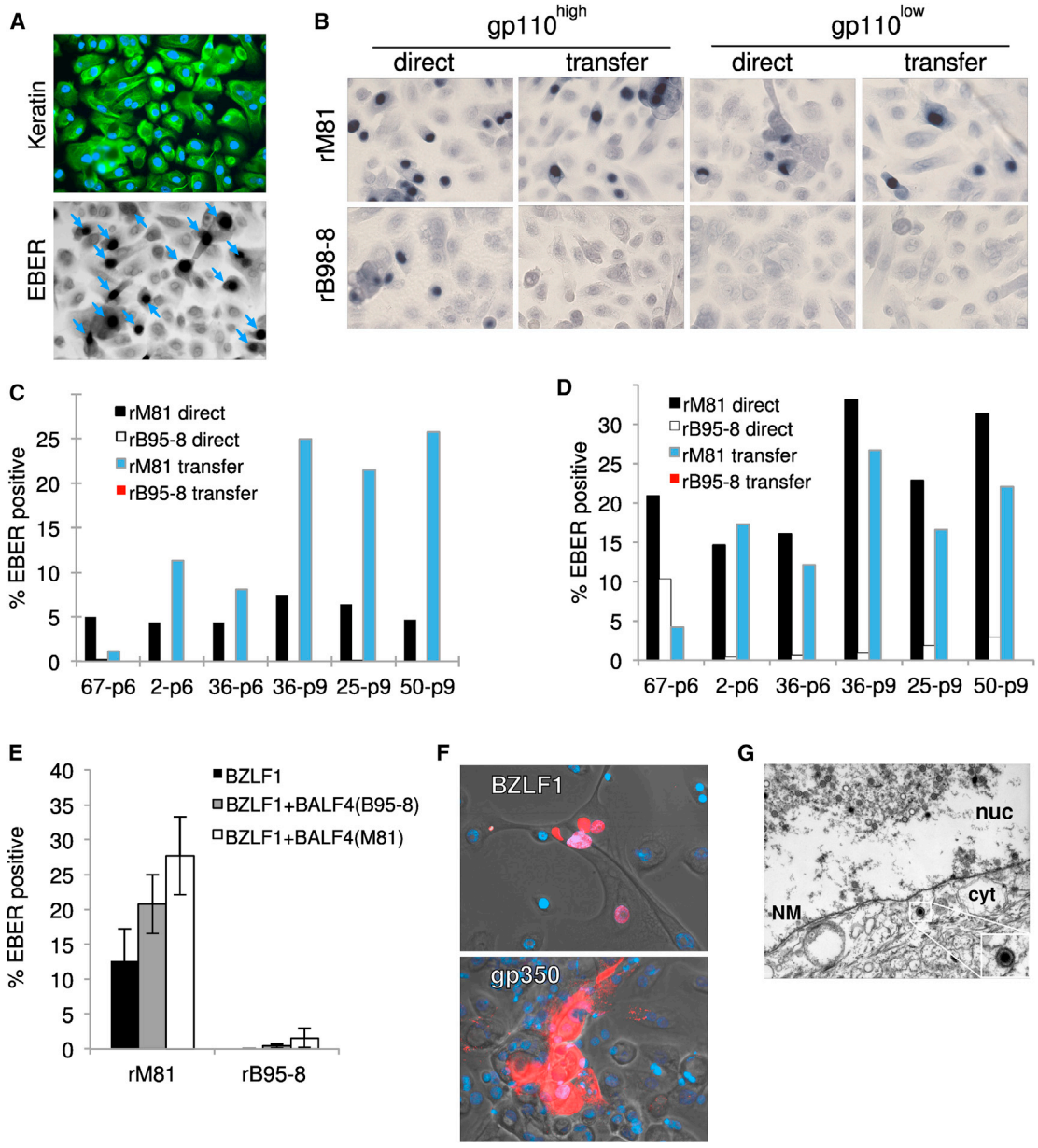

Figure 6. rM81 Infects Primary Epithelial Cells with High Efficiency

(A) Primary epithelial cells were infected with rM81 using transfer infection, stained for pan-keratin expression using a specific antibody coupled to fluorescein isothiocyanate, and counterstained with the nuclear stain Hoechst 33258 (top). The same cells were further evaluated for EBER expression using in situ hybridization (bottom). Infected cells are indicated by blue arrows.

(B) Primary epithelial cells from the same donor (50p9) were submitted to direct or transfer infection with rM81or rB95-8, with or without complementation with gp110. Infected cells were identified by EBER in situ hybridization.

(C) The results of infections performed on multiple primary samples at different passages are compiled in this graph (e.g., 67p6 indicates patient number 67, passage 6). Viruses used in this set of experiments were not complemented with gp110.

(D) The same experiments as in (C) were performed with viruses complemented for gp110 incorporation.

(E) The figure depicts the epitheliotropism of viruses complemented with gp110 encoded by M81 or by B95-8 as determined by EBER expression. Epithelial cells were infected by transfer infection. Values represent mean ± SD.

(F) Epithelial cells infected with rM81 were immunostained for BZLF1 or gp350 (red signals) and nuclei counterstained with Hoechst 33258 (blue staining). Infections were repeated three times with cells from one donor.

(G) Primary epithelial cells infected with rM81 were analyzed by transmission electron microscopy. Nuc, nucleus; NM, nuclear membrane; cyt, cytoplasm.

that are endowed with the ability to replicate at high level in the absence of overt immunosuppression and that are obviously epitheliotropic. Indeed, these patients display very high levels of antibodies against EBV replication proteins whose presence is a predictive factor for NPC development (Cao et al., 2011; Chien et al., 2001; Hsu et al., 2009). It is remarkable that these two defining features are characteristics of M81 and by extension probably of viruses found in NPC that share a considerable degree of homology at the protein level. The excessive intake of substances that activate lytic replication, such as phorbol esters that are abundant in some food frequently consumed in Southeast Asia, could further enhance lytic replication in infected individuals (Davies et al., 1991; Fang et al., 2009; zur Hausen et al., 1978). Altogether, our data make M81-type viruses prime suspects, probably in combination with other cocarcinogens such as smoking and food carcinogens, for the development of NPC.

Virus species can possess a variable number of subtypes. Numerous subtypes of influenza A viruses (Webster et al., 1992) or papillomaviruses (de Villiers et al., 2004) are known. The definition of a subtype typically rests on sequence heterogeneity that might or might not be associated with different properties of the viruses or their ability to cause disease. The division of influenza A viruses in subtypes is justified by the absence of cross-protective immune response between them but also by the ability of some subtypes to cause serious disease. In the case of human papillomavirus, only a restricted number of subtypes cause cervical cancer, and identification of these subtypes has become an important diagnostic tool in the evaluation of chronic cervical infection (de Villiers et al., 2004). Herpesviridae comprise multiple genera and species, but these usually do not have any subtypes (Roizman and Pellett, 2007). We suggest to revise this view in EBV's case and to define M81-type viruses as a particular subtype with a different cell tropism and a preference for lytic replication.

Sequencing of M81 revealed multiple polymorphisms that distinguish the M81 type from other viral strains. Whole-genome sequencing methods will deliver additional information on the sequence of a large number of EBV isolates present in various tumors and will undoubtedly massively increase the diversity of virus sequences. The present report suggests that it will be indispensable to clone these isolates as recombinant viruses in order to understand the impact of polymorphisms on the viral functions, in particular in their ability to induce disease. Indeed, many diseases or tumors associated with EBV such as chronic active infection, gastric carcinoma, BL, nasal NK/T cell lymphomas, and peripheral T cell lymphomas are mainly seen in particular countries or areas (Rickinson and Kieff, 2007; Young and Rickinson, 2004). Our work suggests that particular alleles in genetic elements encoded by these viruses contribute to the development of these diseases, but only a genetic approach will allow a full appreciation of their role.

These observations also suggest that immunization strategies should take into account the genetic diversity within EBV subtypes. Indeed, vaccines generated with epitopes from Western strains might not prevent infection with M81-type strains. Particular attention should be paid to the polymorphisms displayed by late glycoproteins, because these are likely to mediate M81's epitheliotropism.

EXPERIMENTAL PROCEDURES

Cell Lines

All cells were routinely cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Biochrom), and primary B cells were supplemented with 20% FBS until establishment. Primary epithelial cells isolated from normal sphenoidal sinus biopsy material were cultured in keratinocyte serum-free growth medium (KGM-SFM; Invitrogen) as described previously (Feederle et al., 2007). The ethics committee of the University of Heidelberg approved the study (approval 392/2005).

Recombinant Viruses

M81 was cloned onto an F-plasmid as previously described (Delecluse et al., 1998). Viral recombinants were constructed using homologous recombination either with linear targeting vectors or using chromosomal building (Feederle et al., 2010). Details on the constructions of the different recombinants used throughout this study can be found in the Supplemental Information.

Viral Genome Sequencing

The whole-genome map of M81 strain was determined by deep sequencing with an Illumina HiSeq 2000 and assembled using the GS Reference Mapper software. The sequences of the ambiguous regions and the repeat regions were further confirmed using a standard Sanger sequencing method. The information on the M81 genome is available (GenBank accession number KF373730).

Plasmids

A *BZLF1* expression plasmid (p509) was used for the induction of the lytic cycle and the expression plasmid coding *BALF4* derived from B95-8 (p2354) was used for gp110 overexpression (Neuhierl et al., 2009). A similar expression plasmid expressing the M81 BALF4 derived from M81 genome was constructed (B809). Expression plasmids encoding the BLLF1 gene (gp350), either from M81 or B95-8, were constructed into pcDNA3.1 (B702 and B703). The plasmid B735 contains a minimal CMV promoter controlled by a bidirectional TetO operator, a tetracycline transactivator protein (Tet-On) driven by a CAG promoter, the latent EBV origin of replication oriP derived from B95-8, and a rat CD2 gene driven by SV40 promoter (Bornkamm et al., 2005). The *BZLF1* genes from M81 or B95-8 were placed under the tet promoter (B734 and B733).

BZLF1 Expression in LCLs

LCLs generated with the ΔBZLF1 virus were stably transfected with these expression plasmids. Three days postelectroporation, cells carrying the plasmid were enriched by immunopurification using the monoclonal antibody OX34 that binds to CD2 positive cells. A secondary antibody coupled to anti-mouse immunoglobulin G Dynabeads was used to purify CD2-positive cells (Invitrogen).

Bioinformatics

We compared the protein and DNA sequence of the following virus isolates: GD1 (accession number AY961628.3), GD2 (accession number HQ020558.1), HKNPC1 (accession number JQ009376.1), Akata (accession number KC207813.1), Mutu (accession number KC207814.1), and B95-8 (accession number NC_007605). The alignments and distance trees were computed with ClustalW version 2.0.12 and the neighbor joining method using default parameters (Larkin et al., 2007). Distance trees were visualized with the Drawtree program from Joe Felsenstein's Phylip package. The phylogenetic analysis of individual EBV proteins was performed with ClustalW version 2.0.12 for proteins.

Viral Infections

Peripheral blood B cells from several donors were infected with supernatants from virus producer cell lines at room temperature using various MOIs as

determined by qPCR or immunostaining with an antibody specific to EBNA2. Transfer infections were performed as previously described (Shannon-Lowe et al., 2006).

Electron Microscopy

Infected cell lines, primary cells, or pelleted viruses from 6 ml of viral supernatant centrifuged for 2 hr at 30.000 × *g* were fixed in 2.5% glutaraldehyde for 20 min at 4°C. Further preparation, embedding, and sections were carried out as described previously (Granato et al., 2008). Ultrathin sections were examined by electron microscopy (Zeiss).

Immunostaining and Western Blot

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were permeabilized in PBS 0.5% Triton X-100 for 2 min except for samples stained for viral glycoproteins (gp350/220 and VCA). Cells were incubated with the first antibody for 30 min, washed in PBS three times, and incubated with a secondary antibody conjugated to Cy-3 or Alexa 488 for 30 min. Nuclei were counterstained for 1 min with Hoechst 33258 before embedding in 90% glycerol. Western blots for viral glycoproteins have been described before (Neuhierl et al., 2002). The antibodies used are listed in the Supplemental Information.

Southern Blot

Genomic DNA extraction from LCLs, probe preparation and Southern blotting has been described before (Delecluse et al., 1998). In this study, genomic DNAs were digested with BamHI and the blots were hybridized with a ^{32}P-labeled DNA fragment specific to *BMRF1* or to an EBV terminal repeat (Feederle et al., 2009a).

Human Immune System Component Reconstitution in Mice for EBV Infection

Mice with a reconstituted human immune system were generated by intrahepatically injecting newborn NSG-A2 mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(HLA-A2.1)1Enge/SzJ) with human hematopoietic progenitor cells (HPCs) after 1 Gy irradiation (huNSG-A2 mice) (White et al., 2012). Confirmation of successful reconstitution with human immune cells was obtained by analyzing the peripheral blood of the animals approximately 12 weeks after HPC transplantation. The infectious titers of rB95-8 viruses, used for infection of huNSG-A2 mice, were determined by Raji cell infection as Green Raji units (GRU) via GFP expression after infection. In all experiments, 1 × 10^5 GRU of rB95-8 were used for intraperitoneal infection of huNSG-A2 mice. In one experiment, we injected with the same number of rM81 or rB95-8 viral genomes. In the other, we used volumes of rB95-8 and rM81 supernatants that infected primary B cells with the same efficiency, as determined by an EBNA2-specific immunostaining. The infected mice were monitored for 5 weeks postinfection and then euthanized, and their tissues were analyzed by immunohistochemistry. The veterinary office of the canton of Zurich approved the study (approval 148/2011).

Immunohistochemistry

Organs from the euthanized huNSG-A2 mice were fixed in 10% formalin and embedded in paraffin, and 3-μm-thin sections were prepared and immunostained after antigen retrieval (10 mM sodium citrate, 0.05% Tween 20 [pH 6.0]; 98°C for 40 min). Bound antibodies were visualized with the Envision+ Dual link system-HRP (Dako). Pictures were taken with a camera attached to a light microscope (Axioplan, Zeiss).

Statistics

Statistical evaluation was performed with an unpaired t test using two-tailed P values and a confidence interval of 95.

For additional details, refer to the Extended Experimental Procedures.

ACCESSION NUMBERS

The GenBank accession number for the M81 genome sequence is KF373730.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.012.

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